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PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

WEST-WALKER, Gregory, James
A J Park
Huddart Parker Building, 6th floor
1 Post Office Square
P.O. Box 949
Wellington 6015
NOUVELLE-ZÉLANDE

Date of mailing (day/month/year)
12 April 2001 (12.04.01)

Applicant's or agent's file reference
25409 MRB

IMPORTANT NOTIFICATION

International application No.
PCT/NZ99/00227

International filing date (day/month/year)
23 December 1999 (23.12.99)

1. The following indications appeared on record concerning:

[ ] the applicant [ ] the inventor [X] the agent [ ] the common representative

Name and Address

BENNETT, Michael, Roy
West-Walker Bennett
Mobil on the Park
157 Lambton Quay
Wellington
New Zealand

State of Nationality | State of Residence

Telephone No.
64 4 499 9058

Facsimile No.
64 4 499 9306

Teleprinter No.

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[X] the person [X] the name [X] the address [ ] the nationality [ ] the residence

Name and Address

WEST-WALKER, Gregory, James
A J Park
Huddart Parker Building, 6th floor
1 Post Office Square
P.O. Box 949
Wellington 6015
New Zealand

State of Nationality | State of Residence

Telephone No.
64 4 473-8278

Facsimile No.
64 4 472-3358

Teleprinter No.

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[ ] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer
C. Cupello

Telephone No.: (41-22) 338.83.38

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No. PCT/NZ 99/00227

International Filing Date 23 DEC 1999 (23/12/1999)

NEW ZEALAND PATENT OFFICE
P.C.T INTERNATIONAL APPLICATION
Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* 25409 MRB

Box No. I TITLE OF INVENTION

SERINE PROTEASE INHIBITOR

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

THE HORTICULTURE AND FOOD RESEARCH
INSTITUTE OF NEW ZEALAND LIMITED
Batchelar Research Centre
Highway 57
PALMERSTON NORTH
New Zealand

☐ This person is also inventor.

Telephone No.

Facsimile No.

Teleprinter No.

State *(that is, country)* of nationality: New Zealand

State *(that is, country)* of residence: New Zealand

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

SCOTTI, Paul Douglas
872 West Coast Road
Waiatarua, Auckland
New Zealand

This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: New Zealand/~~United States~~

State *(that is, country)* of residence: New Zealand

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

BENNETT, Michael Roy; WEST-WALKER, Gregory James;
RUTLEDGE, Sue Moira
of WEST-WALKER BENNETT
Mobil on the Park
157 Lambton Quay
Wellington
New Zealand

Telephone No. +64 4 499 9058

Facsimile No. +64 4 499 9306

Teleprinter No.

☐ Address for correspondence: Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Continuation of Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

If none of the following spaces is used, this sheet should not be included with the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

DEARING, Sally Caroline
927A Aririmu Road
Aririmu, Auckland
New Zealand

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: New Zealand ~~United Kingdom~~

State *(that is, country)* of residence: New Zealand

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [X] the United States of America only
- [ ] the States indicated in the Supplemental Box

ELECTED 30/NZ

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

GREENWOOD, David Roger
22 Panapa Drive
St John's Park
Auckland
New Zealand

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: New Zealand

State *(that is, country)* of residence: New Zealand

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [X] the United States of America only
- [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

NEWCOMB, Richard David
46 Minnehaha Avenue
Titirangi, Auckland
New Zealand

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: New Zealand

State *(that is, country)* of residence: New Zealand

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [X] the United States of America only
- [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- [ ] applicant only
- [ ] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [ ] the United States of America only
- [ ] the States indicated in the Supplemental Box

- [ ] Further applicants and/or (further) inventors are indicated on another continuation sheet.

Box No.V DESIGNATION OF STAT

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes; at least one must be marked)*:

Regional Patent

- ☒ AP ARIPO Patent: GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, SD Sudan, SZ Swaziland, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- ☒ EA Eurasian Patent: AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- ☒ EP European Patent: AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, CY Cyprus, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- ☒ OA OAPI Patent: BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, GW Guinea-Bissau, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

- ☒ AL Albania
- ☒ AM Armenia
- ☒ AT Austria
- ☒ AU Australia
- ☒ AZ Azerbaijan
- ☒ BA Bosnia and Herzegovina
- ☒ BB Barbados
- ☒ BG Bulgaria
- ☒ BR Brazil
- ☒ BY Belarus
- ☒ CA Canada
- ☒ CH and LI Switzerland and Liechtenstein
- ☒ CN China
- ☒ CU Cuba
- ☒ CZ Czech Republic
- ☒ DE Germany
- ☒ DK Denmark
- ☒ EE Estonia
- ☒ ES Spain
- ☒ FI Finland
- ☒ GB United Kingdom
- ☒ GD Grenada
- ☒ GE Georgia
- ☒ GH Ghana
- ☒ GM Gambia
- ☒ HR Croatia
- ☒ HU Hungary
- ☒ ID Indonesia
- ☒ IL Israel
- ☒ IN India
- ☒ IS Iceland
- ☒ JP Japan
- ☒ KE Kenya
- ☒ KG Kyrgyzstan
- ☒ KP Democratic People's Republic of Korea
- ☒ KR Republic of Korea
- ☒ KZ Kazakhstan
- ☒ LC Saint Lucia
- ☒ LK Sri Lanka
- ☒ LR Liberia
- ☒ LS Lesotho
- ☒ LT Lithuania
- ☒ LU Luxembourg
- ☒ LV Latvia
- ☒ MD Republic of Moldova
- ☒ MG Madagascar
- ☒ MK The former Yugoslav Republic of Macedonia
- ☒ MA Morocco
- ☒ MN Mongolia
- ☒ MW Malawi
- ☒ MX Mexico
- ☒ NO Norway
- ☒ NZ New Zealand
- ☒ PL Poland
- ☒ PT Portugal
- ☒ RO Romania
- ☒ RU Russian Federation
- ☒ SD Sudan
- ☒ SE Sweden
- ☒ SG Singapore
- ☒ SI Slovenia
- ☒ SK Slovakia
- ☒ SL Sierra Leone
- ☒ TJ Tajikistan
- ☒ TM Turkmenistan
- ☒ TR Turkey
- ☒ TT Trinidad and Tobago
- ☒ UA Ukraine
- ☒ UG Uganda
- ☒ US United States of America
- ☒ TZ United Republic of Tanzania
- ☒ UZ Uzbekistan
- ☒ VN Viet Nam
- ☒ YU Yugoslavia
- ☒ ZW Zimbabwe

Check-boxes reserved for designating States (for the purposes of a national patent) which have become party to the PCT after issuance of this sheet:

- ☒ Dominica (DM)
- ☒ South Africa (ZA)
- ☒ United Arab Emirates (AE)
- ☒ Costa Rica (CR)

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)*

Box No. VI PRIORITY CLAIM ☐ Further priority claims as indicated in the Supplemental Box.

Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is: national application: country	regional application:* regional Office	international application: receiving Office
item (1) (23/12/1998) 23 December 1998	NZ 333568	New Zealand		
item (2) (23/07/1999) 23 July 1999	NZ 336906	New Zealand		
item (3)				

☒ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office)* identified above as item(s): (1),(2)

* *Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.*

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used)*: ISA / AU

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority)*: Date *(day/month/year)* Number Country *(or regional Office)*

Box No. VIII CHECK LIST; LANGUAGE OF FILING

This international application contains the following number of sheets:

request	4
description (excluding sequence listing part)	23
claims	3
abstract	1
drawings	4
sequence listing part of description	8
Total number of sheets	43

This international application is accompanied by the item(s) marked below:

1. ☒ fee calculation sheet
2. ☐ separate signed power of attorney
3. ☐ copy of general power of attorney; reference number, if any:
4. ☐ statement explaining lack of signature
5. ☐ priority document(s) identified in Box No. VI as item(s):
6. ☐ translation of international application into (language):
7. ☐ separate indications concerning deposited microorganism or other biological material
8. ☒ nucleotide and/or amino acid sequence listing in computer readable form
9. ☐ other *(specify)*:

Figure of the drawings which should accompany the abstract:

Language of filing of the international application: Language

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

MICHAEL ROY BENNETT
Agent for the Applicant

For receiving Office use only

1. Date of actual receipt of the purported international application:
2. Drawings: ☐ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:
4. Date of timely receipt of the required corrections under PCT Article 11(2):
5. International Searching Authority (if two or more are competent): ISA /
6. ☐ Transmittal of search copy delayed until search fee is paid.

For International Bureau use only

Date of receipt of the record copy by the International Bureau:

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

14

Applicant's or agent's file reference 25409 MRB	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416).
International Application No. **PCT/NZ99/00227**	International Filing Date *(day/month/year)* 23 December 1999	Priority Date *(day/month/year)* 23 December 1998

International Patent Classification (IPC) or national classification and IPC

Int. Cl. [7] C07K 14/81; C07H 21/04; C12N 15/66, 15/70, 15/74, 15/79, 15/81, 1/21, 1/19; A61K 38/55, 38/57; A61P 7/02; A23J 1/04

Applicant

THE HORTICULTURE AND FOOD RESEARCH INSTITUTE OF NEW ZEALAND LIMITED et al

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 3 sheets, including this cover sheet.

☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of sheet(s).

3. This report contains indications relating to the following items:

I	☒	Basis of the report
II	☐	Priority
III	☐	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Lack of unity of invention
V	☒	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☒	Certain documents cited
VII	☐	Certain defects in the international application
VIII	☐	Certain observations on the international application

Date of submission of the demand	Date of completion of the report
13 July 2000	21 December 2000
Name and mailing address of the IPEA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized Officer I Dowd **IAN DOWD** Telephone No. (02) 6283 2273

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/NZ99/00227

I. Basis of the report

1. With regard to the **elements** of the international application:*

- [X] the international application as originally filed.
- [ ] the description, pages , as originally filed,
  pages , filed with the demand,
  pages , received on with the letter of
- [ ] the claims, pages , as originally filed,
  pages , as amended (together with any statement) under Article 19,
  pages , filed with the demand,
  pages , received on with the letter of
- [ ] the drawings, pages , as originally filed,
  pages , filed with the demand,
  pages , received on with the letter of
- [ ] the sequence listing part of the description:
  pages , as originally filed
  pages , filed with the demand
  pages , received on with the letter of

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language which is:

- [ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).
- [ ] the language of publication of the international application (under Rule 48.3(b)).
- [ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, was on the basis of the sequence listing:

- [X] contained in the international application in written form.
- [ ] filed together with the international application in computer readable form.
- [ ] furnished subsequently to this Authority in written form.
- [ ] furnished subsequently to this Authority in computer readable form.
- [ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- [ ] The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

4. [ ] The amendments have resulted in the cancellation of:
   - [ ] the description, pages
   - [ ] the claims, Nos.
   - [ ] the drawings, sheets/fig.

5. [ ] This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* *Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

** *Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/NZ99/00227

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims **1-20, 24**	**YES**
	Claims **21-23**	**NO**
Inventive step (IS)	Claims **1-20, 24**	**YES**
	Claims **21-23**	**NO**
Industrial applicability (IA)	Claims **1-24**	**YES**
	Claims	**NO**

2. Citations and explanations (Rule 70.7)

The Search Report identified the following citations.

D1- STN Medline On-line Abstract Accession No. 1999206055

D2- Chemical Abstracts 130:48310

D3- STN Medline On-line Abstract Accession No.86220028

D4- STN Medline On-line Abstract PMID: 2861005

D5- Chemical Abstracts 94: 62091

D6- Derwent Abstract Accession No. 87230

D7- Derwent Abstract Accession No. 94-163935/20

D8- Derwent Abstract Accession No. 94-163936/20

D9- Derwent Abstract Accession No.79466

D1 disclosed sequences of metal-binding proteins (eg. Figs 1 and 7). But these sequences are different to those of the proteins of the present application in that they do not include any one of the claimed Sequences ID 1 to 5 or has a molecular weight of about 55kDa. Moreover, they are from the blue sea mussel ***Mytilus edulis***. While present claim 8 (vis a-vis claims 9-11) is not restricted to any particular species it is restricted to the earlier-mentioned sequences.

Similar consideration applies with regard to **D2**.

Accordingly, the claimed subject matter is novel and involved inventive step in the light of **D1** and **D2**.

D3, **D4** and **D5** do not disclosed amino acid or nucleotide sequences and relate to different mussel species.

Accordingly, the claimed subject matter is novel and involves inventive step in the light of **D3**, **D4**, and **D5**.

D6-D9 disclosed methods of preparing polypeptide fractions. When once the process of extracting one protein from shellfish and mussels is known then applying it to the proteins or peptide fractions from the species ***Perna canaliculus*** as presently claimed in claims 21-23 is neither novel nor involve inventive step in the light **D6-D9**.

The claimed subject matter is industrially applicable because of the purported uses thereof.

PATENT COOPERATION TREATY

From the:
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

WEST-WALKER BENNETT
PO Box 1344
WELLINGTON
New Zealand

PCT

NOTIFICATION OF TRANSMITTAL OF INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of mailing *day/month/year* 13 FEB 2001

Applicant's or agent's file reference
25409 MRB

IMPORTANT NOTIFICATION

International Application No.	International Filing Date	Priority Date
PCT/NZ99/00227	23 December 1999	23 December 1998

Applicant
THE HORTICULTURE AND FOOD RESEARCH INSTITUTE OF NEW ZEALAND LIMITED et al

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translations to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices)(Article 39(1))(see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide

Name and mailing address of the IPEA/AU	Authorized officer
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	IAN DOWD Telephone No. (02) 6283 2273

PATENT COOPERATION TREATY

From the:
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:	**PCT**
WEST-WALKER BENNETT PO Box 1344 WELLINGTON New Zealand	WRITTEN OPINION (PCT Rule 66)
	Date of mailing *(day/month/year)* 4 August 2000
Applicant's or agent's file reference 25409 MRB	**REPLY DUE** within **TWO MONTHS** from the above date of mailing

International application No.	International filing date *(day/month/year)*	Priority Date *(day/month/year)*
PCT/NZ99/00227	23 December 1999	23 December 1998

International Patent Classification (IPC) or both national classification and IPC

Int. Cl. [7] C07K 14/81, C07H 21/04, C12N 15/66, 15/70, 15/74, 15/79, 15/81, 1/21, 1/19, A61K 38/55, 38/57, A61P 7/02, A23J 1/04

Applicant

THE HORTICULTURE AND FOOD RESEARCH INSTITUTE OF NEW ZEALANDLIMITED et al

1. This written opinion is the **first** drawn by this International Preliminary Examining Authority.
2. This opinion contains indications relating to the following items:.

I	[X]	Basis of the opinion
II	[ ]	Priority
III	[ ]	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	[ ]	Lack of unity of invention
V	[X]	Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	[X]	Certain documents cited
VII	[ ]	Certain defects in the international application
VIII	[ ]	Certain observations on the international application

3. The applicant is hereby **invited to reply** to this opinion.

When? See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also For an additional opportunity to submit amendments, see Rule 66.4. For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4*bis*. For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: **23 April 2001**

Name and mailing address of the IPEA/AU	Authorized Officer
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	**S.R. IDRUS** Telephone No. (02) 6283 2536

Form PCT/IPEA/408 (Cover sheet) (July 1998)

WRITTEN OPINION

International application No.
PCT/NZ99/00227

I. Basis of the opinion

1. With regard to the **elements** of the international application:*

[X] the international application as originally filed.

[ ] the description, pages , as originally filed,
pages , filed with the demand,
pages , received on with the letter of

[ ] the claims, pages , as originally filed,
pages , as amended under Article 19,
pages , filed with the demand,
pages , received on with the letter of

[ ] the drawings, pages , as originally filed,
pages , filed with the demand,
pages , received on with the letter of

[ ] the sequence listing part of the description:
pages , as originally filed
pages , filed with the demand
pages , received on with the letter of

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language which is:

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application (under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the written opinion was drawn on the basis of the sequence listing:

[X] contained in the international application in printed form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. [ ] The amendments have resulted in the cancellation of:

[ ] the description, pages

[ ] the claims, Nos.

[ ] the drawings, sheets/fig.

5. [ ] This opinion has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed"*

WRITTEN [redacted]NION

International application No.
PCT/NZ99/00227

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	**1-20, 24**	**YES**
	Claims	**21-23**	**NO**
Inventive step (IS)	Claims	**1-20, 24**	**YES**
	Claims	**21-23**	**NO**
Industrial applicability (IA)	Claims	**1-24**	**YES**
	Claims		**NO**

2. Citations and explanations

The Search Report identified the following citations.

D1- STN Medline On-line Abstract Accession No. 1999206055

D2- Chemical Abstracts 130:48310

D3- STN Medline On-line Abstract Accession No.86220028

D4- STN Medline On-line Abstract PMID: 2861005

D5- Chemical Abstracts 94: 62091

D6- Derwent Abstract Accession No. 87230

D7- Derwent Abstract Accession No. 94-163935/20

D8- Derwent Abstract Accession No. 94-163936/20

D9- Derwent Abstract Accession No.79466

D1 disclosed sequences of metal-binding proteins (eg. Figs 1 and 7). But these sequences are different to those of the proteins of the present application in that they do not include any one of the claimed Sequences ID 1 to 5 or has a molecular weight of about 55kDa. Moreover, they are from the blue sea mussel ***Mytilus edulis***. While present claim 8 (vis a-vis claims 9-11) is not restricted to any particular species it is restricted to the earlier-mentioned sequences.

Similar consideration applies with regard to **D2**.

Accordingly, the claimed subject matter is novel and involved inventive step in the light of **D1** and **D2**.

D3, **D4** and **D5** do not disclosed amino acid or nucleotide sequences and relate to different mussel species.

Accordingly, the claimed subject matter is novel and involves inventive step in the light of **D3**, **D4**, and **D5**.

D6-D9 disclosed methods of preparing polypeptide fractions. When once the process of extracting one protein from shellfish and mussels is known then applying it to the proteins or peptide fractions from the species ***Perna canaliculus*** as presently claimed in claims 21-23 is neither novel nor involve inventive step in the light **D6-D9**.

The claimed subject matter is industrially applicable because of the purported uses thereof.

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

Application No. Patent No.	Publication date *(day/month/year)*	Filing date *(day/month/year)*	Priority date (valid claim) *(day/month/year)*

D1- STN Medline On-line Abstract Accession No. 1999206055

Please see comments made in relation to this citation in Box V, previously.

2. Non-written disclosures (Rule 70.9)

Kind of non-written disclosure	Date of non-written disclosure *(day/month/year)*	Date of written disclosure referring to non-written disclosure *(day/month/year)*

The demand must be filed directly with the competent International Preliminary Examining Authority, if two or more Authorities are competent, with the one chosen by the applicant. The name or two-letter code of that Authority may be indicated by the applicant on the line below:

IPEA/ _____

PCT

CHAPTER II

DEMAND

under Article 31 of the Patent Cooperation Treaty:
The undersigned requests that the international application specified below be the subject of international preliminary examination according to the Patent Cooperation Treaty and hereby elects all eligible States (except where otherwise indicated).

For International Preliminary Examining Authority use only

Identification of IPEA	Date of receipt of DEMAND

Box No. I IDENTIFICATION OF THE INTERNATIONAL APPLICATION

Applicant's or agent's file reference: 25409 MRB

International application No.	International filing date *(day/month/year)*	(Earliest) Priority date *(day/month/year)*
PCT/NZ99/00227	23 December 1999 (23/12/1999)	23 December 1998 (23/12/1998)

Title of invention: SERINE PROTEASE INHIBITOR

Box No. II APPLICANT(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

THE HORTICULTURE AND FOOD RESEARCH
INSTITUTE OF NEW ZEALAND LIMITED
Batchelar Research Centre
Highway 57
Palmerston North
New Zealand

Telephone No.:

Facsimile No.:

Teleprinter No.:

State *(that is, country)* of nationality:	State *(that is, country)* of residence:
New Zealand	New Zealand

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

SCOTTI, Paul Douglas
872 West Coast Road
Waiatarua, Auckland
New Zealand

State *(that is, country)* of nationality:	State *(that is, country)* of residence:
New Zealand	New Zealand

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

DEARING, Sally Caroline
927A Ariarimu Road
Ariarimu, Auckland
New Zealand

State *(that is, country)* of nationality:	State *(that is, country)* of residence:
New Zealand	New Zealand

[X] Further applicants are indicated on a continuation sheet.

Sheet No. 2

International application No. PCT/NZ99/00227

Continuation of Box No. II APPLICANT(S)

If none of the following sub-boxes is used, this sheet should not be included in the demand.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

GREENWOOD, David Roger
22 Panapa Drive
St John's Park
Auckland
New Zealand

State *(that is, country)* of nationality: New Zealand

State *(that is, country)* of residence: New Zealand

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

NEWCOMB, Richard David
46 Minnehaha Avenue
Titirangi, Auckland
New Zealand

State *(that is, country)* of nationality: New Zealand

State *(that is, country)* of residence: New Zealand

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

☐ Further applicants are indicated on another continuation sheet.

International application No.
PCT/NZ99/00227

Box No. III AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The following person is [X] agent [ ] common representative

and [X] has been appointed earlier and represents the applicant(s) also for international preliminary examination.

[ ] is hereby appointed and any earlier appointment of (an) agent(s)/common representative is hereby revoked.

[ ] is hereby appointed, specifically for the procedure before the International Preliminary Examining Authority, in addition to the agent(s)/common representative appointed earlier.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

BENNETT, Michael Roy; RUTLEDGE, Sue Moira
WEST-WALKER, Gregory James
of WEST-WALKER BENNETT
Mobil on the Park
157 Lambton Quay
Wellington
NEW ZEALAND

Telephone No.: 64 4 499 9058

Facsimile No.: 64 4 499 9306

Teleprinter No.:

[ ] **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Box No. IV BASIS FOR INTERNATIONAL PRELIMINARY EXAMINATION

Statement concerning amendments:*

1. The applicant wishes the international preliminary examination to start on the basis of:

[X] the international application as originally filed

the description [X] as originally filed
[ ] as amended under Article 34

the claims [X] as originally filed
[ ] as amended under Article 19 (together with any accompanying statement)
[ ] as amended under Article 34

the drawings [X] as originally filed
[ ] as amended under Article 34

2. [ ] The applicant wishes any amendment to the claims under Article 19 to be considered as reversed.

3. [ ] The applicant wishes the start of the international preliminary examination to be postponed until the expiration of 20 months from the priority date unless the International Preliminary Examining Authority receives a copy of any amendments made under Article 19 or a notice from the applicant that he does not wish to make such amendments (Rule 69.1(d)). *(This check-box may be marked only where the time limit under Article 19 has not yet expired.)*

* Where no check-box is marked, international preliminary examination will start on the basis of the international application as originally filed or, where a copy of amendments to the claims under Article 19 and/or amendments of the international application under Article 34 are received by the International Preliminary Examining Authority before it has begun to draw up a written opinion or the international preliminary examination report, as so amended.

Language for the purposes of international preliminary examination: English

[X] which is the language in which the international application was filed.

[ ] which is the language of a translation furnished for the purposes of international search.

[ ] which is the language of publication of the international application.

[ ] which is the language of the translation (to be) furnished for the purposes of international preliminary examination.

Box No. V ELECTION OF STATES

The applicant hereby elects all eligible States *(that is, all States which have been designated and which are bound by Chapter II of the PCT)*

excluding the following States which the applicant wishes not to elect:

Sheet No. 4

International application No.
PCT/NZ99/00227

Box No. VI CHECK LIST

The demand is accompanied by the following elements, in the language referred to in Box No. IV, for the purposes of international preliminary examination:			For International Preliminary Examining Authority use only: received	not received
1. translation of international application	:	sheets	☐	☐
2. amendments under Article 34	:	sheets	☐	☐
3. copy (or, where required, translation) of amendments under Article 19	:	sheets	☐	☐
4. copy (or, where required, translation) of statement under Article 19	:	sheets	☐	☐
5. letter	:	sheets	☐	☐
6. other *(specify)*	:	sheets	☐	☐

The demand is also accompanied by the item(s) marked below:

1. [X] fee calculation sheet
2. [ ] separate signed power of attorney
3. [ ] copy of general power of attorney; reference number, if any:
4. [ ] statement explaining lack of signature
5. [ ] nucleotide and or amino acid sequence listing in computer readable form
6. [ ] other *(specify)*:

Box No. VII SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the demand).

MICHAEL ROY BENNETT
Agent for the Applicants

For International Preliminary Examining Authority use only

1. Date of actual receipt of DEMAND:
2. Adjusted date of receipt of demand due to CORRECTIONS under Rule 60.1(b):
3. [ ] The date of receipt of the demand is AFTER the expiration of 19 months from the priority date and item 4 or 5, below, does not apply. [ ] The applicant has been informed accordingly.
4. [ ] The date of receipt of the demand is WITHIN the period of 19 months from the priority date as extended by virtue of Rule 80.5.
5. [ ] Although the date of receipt of the demand is after the expiration of 19 months from the priority date, the delay in arrival is EXCUSED pursuant to Rule 82.

For International Bureau use only

Demand received from IPEA on:

CHAPTER II

PCT

FEE CALCULATION SHEET

Annex to the Demand for international preliminary examination

International application No. PCT/NZ99/00227	For International Preliminary Examining Authority use only
Applicant's or agent's file reference 25409 MRB	Date stamp of the IPEA

Applicant

THE HORTICULTURE AND FOOD RESEARCH INSTITUTE OF NEW ZEALAND LIMITED

Calculation of prescribed fees

1. Preliminary examination fee	AUD450.00	P
2. Handling fee *(Applicants from certain States are entitled to a reduction of 75% of the handling fee. Where the applicant is (or all applicants are) so entitled, the amount to be entered at H is 25% of the handling fee.)*	AUD238.00	H
3. Total of prescribed fees Add the amounts entered at P and H and enter total in the TOTAL box	AUD688.00	TOTAL

Mode of Payment

☐	authorization to charge deposit account with the IPEA (see below)	☐	cash
☐	cheque	☐	revenue stamps
☐	postal money order	☐	coupons
☒	bank draft	☐	other *(specify)*:

Deposit Account Authorization *(this mode of payment may not be available at all IPEAs)*

The IPEA/ _____ ☐ is hereby authorized to charge the total fees indicated above to my deposit account.

☐ *(this check-box may be marked only if the conditions for deposit accounts of the IPEA so permit)* is hereby authorized to charge any deficiency or credit any overpayment in the total fees indicated above to my deposit account.

Deposit Account Number _____ Date *(day/month/year)* _____ Signature _____

See Notes to the fee calculation sheet

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
25409 MRB		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/NZ99/00227	23 December 1999	23 December 1998

Applicant

THE HORTICULTURE AND FOOD RESEARCH INSTITUTE OF NEW ZEALANDLIMITED

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 6 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing:

   [X] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readable form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (See Box II).

4. With regard to the **title**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

   [ ] the text is approved as submitted by the applicant

   [X] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No.

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure

   [ ] because this figure better characterizes the invention

   [X] None of the figures

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
C07K 14/81, C07H 21/04, C12N 15/66, 15/70, 15/74, 15/79, 15/81, 1/21, 1/19, A61K 38/55, 38/57, A61P 7/02, A23J 1/04

A1

(11) International Publication Number: WO 00/39165

(43) International Publication Date: 6 July 2000 (06.07.00)

(21) International Application Number: PCT/NZ99/00227

(22) International Filing Date: 23 December 1999 (23.12.99)

(30) Priority Data:

333568	23 December 1998 (23.12.98)	NZ
336906	23 July 1999 (23.07.99)	NZ

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(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: SCOTTI, Paul, Douglas [NZ/NZ]; 872 West Coast Road, Waiatarua, Auckland (NZ). DEARING, Sally, Caroline [NZ/NZ]; 927A Aririmu Road, Aririmu, Auckland (NZ). GREENWOOD, David, Roger [NZ/NZ]; 22 Panapa Drive, St. John's Park, Auckland (NZ). NEWCOMB, Richard, David [NZ/NZ]; 46 Minnehaha Avenue, Titirangi, Auckland (NZ).

(74) Agents: BENNETT, Michael, Roy et al.; West-Walker Bennett, Mobil on the Park, 157 Lambton Quay, Wellington (NZ).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: SERINE PROTEASE INHIBITOR

(57) Abstract

The invention provides a protein which exhibits, *inter alia*, anti-thrombin activity and divalent metal cation binding activity. The protein can be readily extracted from the green-lipped mussell, *Perna canaliculus*, and formulated into foodstuffs, nutraceuticals and the like, and has a molecular weight of about 55 kDa and an amino acid sequence which includes one or more of the following: (a) DGEQCNDGQN (SEQ ID NO.1), (b) QGGHEVESERVACCVIGRA (SEQ ID NO. 2), (c) GQSHPEIVH (SEQ ID NO. 3), (d) YHGHDDA (SEQ ID NO. 4), (e) VVNEVHH (SEQ ID NO. 5)

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
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SERINE PROTEASE INHIBITOR

This invention relates to a protein and compositions which contain it. More particularly, it relates to a protein which *inter alia* exhibits activity as a metal cation binding agent and as an anti-thrombin agent.

BACKGROUND

Thrombin is a serine protease involved in blood coagulation. It has specificity for the cleavage of arginine-lysine bonds as well as cleaving an arginine-threonine bond in pro-thrombin, releasing pre-thrombin which is subsequently cleaved to produce active thrombin. This active thrombin can then release more thrombin from pro-thrombin. In blood clotting and coagulation, thrombin cleaves fibrinopeptide B from fibrinogen as well as converting blood factors IX to IXa, V to Va, VIII to VIIIa and XIII to XIIIa.

Inhibitors of thrombin therefore inhibit coagulation and have application in any procedure where coagulation is undesirable. One such application is in the collection and storage of blood products. Another is in medicaments for preventing or reducing coagulation for example in treating or preventing cardiac malfunctions.

Anti-thrombin agents are known. One example is anti-thrombin III (AT-III). However, AT-III is capable of effectively inhibiting thrombin only in the presence of heparin.

The applicants have now identified a novel protein which has a range of activities, including anti-thrombin activity, and which when active against thrombin does not require heparin as a cofactor. It is towards this protein that the present invention is broadly directed.

SUMMARY OF THE INVENTION

In a first aspect, the present invention provides an isolated protein which has a molecular weight of about 55 kDa and an amino acid sequence which includes one or more of the following:

(a) DGEQCNDGQN (SEQ ID NO. 1)

(b) QGGHEVESERVACCVIGRA (SEQ ID NO. 2)

(c) GQSHPEIVH (SEQ ID NO. 3)

(d) YHGHDDA (SEQ ID NO. 4)

(e) VVNEVHH (SEQ ID NO. 5),

or an active fragment thereof.

In a further aspect, the invention provides an isolated protein which comprises the amino acid sequence of

D G E Q C N D G Q N K D D H H D D H H D D H H D D H D D D D
E T M H Y A Q C E M E P N P H M A S S L H H H V H G S I E L
S Q K G H G A V Y L E L H L V G F N T S E D H D D H H H G L
H L H M L G D M S A G C D S I G E L Y N A H P E K H A D P G
D L G D L V D D D R G V V N E V H H Y A W L D I D G T A P N
T E A L I G H S M T I L Q G S H T D A D T P A S R I A C C V
I G H G K A R P E T A A A L H H E L E E D K T E H Y A H C D
V R S N T H Q P K A L H H H V H G T I D F K Q V G Y G D L E
V S Y H L E G F N V S D D H K D H L H D V Q I Y A N G D L T
S G C D N L G A K Y D P H E D Y H S E L G D L G D I H D D D
H G V V N E S H R Y S W I N I F G D D S V L G R S I A I H Q
R D H L H K S A K I A C C V I G R G Q S H P E I V H R A K C
V V R P N T E S T G L H H H V S G S I T F E Q T P G G S T H
M T A D L K G F N V S E D L S H H R H G V Q L H E W G D M S
H G C H S L G R M Y H G H D D A H D P K R P G D L G D V I D
D S H G I V H S T R T F D H L N V E D L N A R S L V I M Q G
G H E V E S E R V A C C V I G R A (SEQ ID NO. 7)

or an active fragment thereof.

In yet a further aspect, the invention provides an isolated protein which is obtainable from the haemolymph of *Perna canaliculus* which has an apparent molecular weight of 75 kda determined by PAGE, or an active fragment thereof.

Conveniently, said protein or fragment has activity as:

(i) a serine protease inhibitor; or

(ii) a divalent cation binding agent.

The invention further provides a protein which is a functionally equivalent variant of a protein or fragment as defined above.

Still further, the invention provides a protein which is obtainable from a shellfish other than *Perna canaliculus* and which is a functionally equivalent variant of a protein or fragment as defined above.

In another aspect, the invention provides a polynucleotide encoding a protein or fragment as defined above.

The polynucleotide may comprise the nucleotide sequence of

5′ GAYGGGGAGCAGTGTAACGATGGGCAGAACAAAGATGACCACCATGACGA
CCACCACGATGATCACCATGACGACCATGATGATGATGATGAAACAATGCACT
ATGCCCAGTGTGAAATGGAACCAAACCCTCATATGGCTAGCAGCCTTCACCA
CCATGTCCATGGCAGCATAGAGTTGTCACAGAAGGGTCATGGAGCTGTTTAT
CTAGAACTTCATCTTGTCGGATTCAACACAAGTGAAGACCATGACGACCACCA
TCATGGACTTCATCTGCACATGCTTGGTGACATGTCAGCAGGTTGTGATTCTA
TTGGCGAACTGTACAATGCTCACCCAGAAAAACATGCTGACCCTGGTGACCT
CGGTGACCTGGTTGACGATGATAGGGGCGTGGTTAATGAAGTTCATCATTATG
CTTGGTTGGACATTGATGGTACAGCACCAAACACCGAAGCTCTCATTGGACA
CTCAATGACTATTTTACAAGGGAGTCACACCGATGCTGATACCCCAGCCAGTA
GAATCGCCTGTTGTGTTATTGGTCATGGAAAAGCTCGCCCAGAAACAGCAGC
TGCTCTACATCACGAGCTAGAGGAAGATAAAACTGAGCATTATGCCCATTGTG
ACGTAAGATCTAATACACACCAACCAAAGGCTCTTCATCATCATGTCCACGGA
ACCATCGATTTCAAACAAGTTGGTTATGGTGACCTTGAAGTGTCCTACCATTTA
GAGGGATTTAATGTAAGTGATGACCACAAAGATCATCTCCATGACGTACAGAT
CTACGCCAACGGTGACCTGACCAGTGGATGTGATAACCTCGGTGCTAAATAT
GATCCTCATGAAGATTACCACAGTGAGTTGGGTGATCTAGGAGATATTCACGA
TGATGACCATGGCGTTGTCAATGAAAGCCACAGATATTCCTGGATCAATATCT
TCGGTGATGACAGTGTCCTGGGACGTTCTATTGCCATTCACCAAAGAGACCAT
CTTCATAAAAGTGCCAAAATTGCCTGTTGTGTCATAGGACGTGGACAGAGCCA
TCCAGAAATTGTTCACAGAGCTAAATGTGTTGTCAGACCTAATACAGAATCTAC
TGGTTTACATCACCATGTCTCTGGTTCTATAACATTCGAACAGACCCCTGGAG

GATCAACACATATGACGGCTGATCTCAAAGGATTTAACGTTAGTGAGGACTTG
TCACATCATCGTCATGGTGTGCAGCTCCATGAATGGGGAGATATGTCCCATG
GCTGTCACTCCTTAGGCAGAATGTACCATGGTCATGATGATGCTCATGACCCC
AAAAGACCTGGTGACCTTGGTGATGTTATAGATGATTCCCATGGCATCGTTCA
TTCAACTAGAACCTTTGATCATCTTAATGTTGAAGATCTTAACGCACGTTCCCT
TGTGATTATGCAGGGCGGACATGAGGTCGAGAGTGAGAGGGTTGCTTGCTGT
GTTATAGGACGGGCA (SEQ ID NO. 6)

or a variant thereof.

Still further, the invention provides a vector or construct which includes a polynucleotide as defined above.

In another aspect, the invention provides a composition which comprises a protein or fragment as defined above.

The composition may be a medicament, a food, a dietary supplement, (optionally including the protein associated with or bound to at least one divalent cation of dietary significance) or a bioremediation agent.

In still another aspect, the invention provides a process for obtaining a protein as defined above which comprises the step of centrifuging material containing *Perna canaliculus* haemolymph or an extract thereof and recovering the sedimented protein.

DESCRIPTION OF THE DRAWINGS

While the present invention is broadly as defined above, it also includes embodiments of which the following description provides examples. In particular, a better understanding of the present invention will be gained through reference to the accompanying drawings in which

Figure 1: Purification of pernin from mussel haemolymph

a) light-scattering band following centrifugation of *P. canaliculus* haemolymph in CsCl; haemolymph was first centrifuged at low speed to remove

haemocytes and then at high speed; the re-suspended pellet was then centrifuged in CsCl.

b) UV absorption profile (254 nm wavelength) from fractionation of the CsCl gradient; the light-scattering material in figure 1a appears as a peak.

c) protein composition in 1 ml fractions of a CsCl gradient following electrophoresis in a 12% polyacrylamide gel; the heavily stained (Coomassie) bands coincide with the position of the light-scattering and UV-absorbing regions of the gradient; the molecular weight was approximately 75 kDa as compared with polypeptide molecular weight standards (lane 6) (refer Figure 4a for standards). Lanes 1-5 and 7-9 contained samples from the CsCl gradient.

Figure 2: Virus-like particles observed by transmission electron microscopy of material in light scattering band in a CsCl gradient. Bar in micrograph represents 100 nm.

Figure 3: HPLC elution profile of pernin at 280 nm wavelength purified by CsCl gradient centrifugation..

Figure 4: SDS-PAGE profiles (12% gels) of aggregating protein species from *P. canaliculus* and other shellfish species

a) proteins extracted from whole shellfish and purified as described in Materials and Methods: lane 1: molecular weight standards (Bio-Rad, USA) :**pb** phosphorylase B, 97.4 kDa; **bsa** bovine serum albumin, 66 kDa; **ova** ovalbumin, 45 kDa; **ca** carbonic anhydrase, 31 kDa; lane 2: Greenshell™ mussel *P. canaliculus*; lane 3: blue mussel *Mytilis edulis*; lane 4: oyster *Crassostrea gigas*; lane 5: pipis *Paphies australis*.

b) PAGE analysis of human transferrin (Sigma, USA, MW *ca.* 80 kDa), a glycosylated protein, and pernin from *P. canaliculus* following treatment with endoglycosidase-F: lane 1: untreated transferrin; lane 2: transferrin treated

with glycosidase-F; lane 3: untreated pernin lane 4: pernin treated with glycosidase-F.

Figure 5: Activity of *P. canaliculus* haemolymph protein following centrifugation in a 30 kDa molecular weight exclusion filter for 10 min at 1000 *g* (Ultrafree-MC filter, 30,000 MW exclusion, Millipore, USA)

a) SDS-PAGE profile of haemolymph protein at various stages of purification. Lane 1: "crude" haemolymph (haemocytes removed); lane 2: resuspended pellet after ultracentrifugation of "crude" haemolymph for 80 min at 250,000 *g*; lane 3: pernin retentate; lane 4: filtrate (no proteins evident); lane 5: molecular weight markers, (refer Figure 4a); lanes 6,7: 10-fold dilutions of samples from lanes 2 and 3.

b) Anti-thrombin activity of 30,000 MW exclusion filter retentate and filtrate.

con+ = the standard 1/41 dilution of human plasma (i.e. standard anti-thrombin III activity);

con - thrombin with no added plasma (buffer control); **filtrate**: material passed through a 30,000 MW exclusion filter;

retentate: pernin protein retained by exclusion filter.

DESCRIPTION OF THE INVENTION

As broadly outlined above, in one aspect the present invention provides a novel protein. The protein of the invention has an apparent molecular weight of 75 kDa, calculated by polyacrylamide gel electrophoresis (PAGE). The molecular weight inferred from the gene sequence is approximately 55 kDa.

One specific protein of the invention was initially identified as an extract from the New Zealand green lipped mussel *P. canaliculus*. It is therefore obtainable by extraction directly from *P. canaliculus*.

This protein has the amino acid sequence of SEQ ID NO. 7.

The protein of the invention can include its entire native amino acid sequence or can include only parts of that sequence where such parts constitute fragments which remain biologically active (active fragments). Such activity will normally be as a serine protease inhibitor or a divalent cation binding agent but is not restricted to these activities.

The invention also includes within its scope functionally equivalent variants of the protein of SEQ ID NO. 7.

The phrase "functionally equivalent variants" recognises that it is possible to vary the amino acid of a protein while retaining substantially equivalent functionality. For example, a protein can be considered a functional equivalent of another protein for a specific function if the equivalent peptide is immunologically cross-reactive with and has at least substantially the same function as the original protein.

The functionally equivalent protein need not be the same size as the original. The equivalent can be, for example, a fragment of the protein, a fusion of the protein with another protein or carrier, or a fusion of a fragment with additional amino acids. It is also possible to substitute amino acids in a sequence with equivalent amino acids using conventional techniques. Groups of amino acids normally held to be equivalent are:

(a) Ala, Ser, Thr, Pro, Gly;
(b) Asn, Asp, Glu, Gln;
(c) His, Arg, Lys;
(d) Met, Leu, Ile, Val; and
(e) Phe, Tyr, Trp.

Polypeptide sequences may be aligned, and percentage of identical amino acids in a specified region may be determined against another sequence, using computer algorithms that are publicly available. The similarity of polypeptide sequences may be examined using the BLASTP algorithm. BLASTP software is available on the NCBI anonymous FTP server (ftp://ncbi.nlm.nih.gov) under /blast/executables/. The use of the BLAST family of algorithms, including BLASTP, is described at NCBI's website at URL http://www.ncbi.nlm.nih.gov/BLAST/newblast.html and in the publication

of Altschul, Stephen F., *et al.* (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", *Nucleic Acids Res.* 25:3389-34023.

The protein of the invention together with its active fragments and other variants may be generated by synthetic or recombinant means. Synthetic polypeptides having fewer than about 100 amino acids, and generally fewer than about 50 amino acids, may be generated by techniques well known to those of ordinary skill in the art. For example, such peptides may be synthesised using any of the commercially available solid-phase techniques such as the Merryfield solid phase synthesis method, where amino acids are sequentially added to a growing amino acid chain (see Merryfield, J. Am. Chem. Soc 85: 2146-2149 (1963)). Equipment for automative synthesis of peptides is commercially available from suppliers such as Perkin Elmer/Applied Biosystems, Inc. and may be operated according to the manufacturers instructions.

The protein, or a fragment or variant thereof, may also be produced recombinantly by inserting a polynucleotide (usually DNA) sequence that encodes the protein into an expression vector and expressing the protein in an appropriate host. Any of a variety of expression vectors known to those of ordinary skill in the art may be employed. Expression may be achieved in any appropriate host cell that has been transformed or transfected with an expression vector containing a DNA molecule which encodes the recombinant protein. Suitable host cells includes procaryotes, yeasts and higher eukaryotic cells. Preferably, the host cells employed are *E. coli*, yeasts or a mammalian cell line such as COS or CHO, or an insect cell line, such as SF9, using a baculovirus expression vector. The DNA sequence expressed in this matter may encode the naturally occurring protein, fragments of the naturally occurring protein or variants thereof.

DNA sequences encoding the protein or fragments may be obtained by screening an appropriate *P. canaliculus* cDNA or genomic DNA library for DNA sequences that hybridise to degenerate oligonucleotides derived from partial amino acid sequences of the protein. Suitable degenerate oligonucleotides may be designed and synthesised by standard techniques and the screen may be performed as described, for example, in Maniatis *et al.* Molecular Cloning - A Laboratory Manual, Cold Spring Harbour Laboratories, Cold Spring Harbour, NY (1989). The polymerase

chain reaction (PCR) may be employed to isolate a nucleic acid probe from genomic DNA, a cDNA or genomic DNA library. The library screen may then be performed using the isolated probe.

Variants of the protein may be prepared using standard mutagenesis techniques such as oligonucleotide-directed site specific mutagenesis.

A specific polynucleotide of the invention has the nucleotide sequence of SEQ ID NO. 6 as follows:

5′ GAYGGGGAGCAGTGTAACGATGGGCAGAACAAAGATGACCACCATGACGA
CCACCACGATGATCACCATGACGACCATGATGATGATGATGAAACAATGCACT
ATGCCCAGTGTGAAATGGAACCAAACCCTCATATGGCTAGCAGCCTTCACCA
CCATGTCCATGGCAGCATAGAGTTGTCACAGAAGGGTCATGGAGCTGTTTAT
CTAGAACTTCATCTTGTCGGATTCAACACAAGTGAAGACCATGACGACCACCA
TCATGGACTTCATCTGCACATGCTTGGTGACATGTCAGCAGGTTGTGATTCTA
TTGGCGAACTGTACAATGCTCACCCAGAAAAACATGCTGACCCTGGTGACCT
CGGTGACCTGGTTGACGATGATAGGGGCGTGGTTAATGAAGTTCATCATTATG
CTTGGTTGGACATTGATGGTACAGCACCAAACACCGAAGCTCTCATTGGACA
CTCAATGACTATTTTACAAGGGAGTCACACCGATGCTGATACCCCAGCCAGTA
GAATCGCCTGTTGTGTTATTGGTCATGGAAAAGCTCGCCCAGAAACAGCAGC
TGCTCTACATCACGAGCTAGAGGAAGATAAAACTGAGCATTATGCCCATTGTG
ACGTAAGATCTAATACACACCAACCAAAGGCTCTTCATCATCATGTCCACGGA
ACCATCGATTTCAAACAAGTTGGTTATGGTGACCTTGAAGTGTCCTACCATTTA
GAGGGATTTAATGTAAGTGATGACCACAAAGATCATCTCCATGACGTACAGAT
CTACGCCAACGGTGACCTGACCAGTGGATGTGATAACCTCGGTGCTAAATAT
GATCCTCATGAAGATTACCACAGTGAGTTGGGTGATCTAGGAGATATTCACGA
TGATGACCATGGCGTTGTCAATGAAAGCCACAGATATTCCTGGATCAATATCT
TCGGTGATGACAGTGTCCTGGGACGTTCTATTGCCATTCACCAAAGAGACCAT
CTTCATAAAAGTGCCAAAATTGCCTGTTGTGTCATAGGACGTGGACAGAGCCA
TCCAGAAATTGTTCACAGAGCTAAATGTGTTGTCAGACCTAATACAGAATCTAC
TGGTTTACATCACCATGTCTCTGGTTCTATAACATTCGAACAGACCCCTGGAG
GATCAACACATATGACGGCTGATCTCAAAGGATTTAACGTTAGTGAGGACTTG
TCACATCATCGTCATGGTGTGCAGCTCCATGAATGGGGAGATATGTCCCATG
GCTGTCACTCCTTAGGCAGAATGTACCATGGTCATGATGATGCTCATGACCCC
AAAAGACCTGGTGACCTTGGTGATGTTATAGATGATTCCCATGGCATCGTTCA

TTCAACTAGAACCTTTGATCATCTTAATGTTGAAGATCTTAACGCACGTTCCCT
TGTGATTATGCAGGGCGGACATGAGGTCGAGAGTGAGAGGGTTGCTTGCTGT
GTTATAGGACGGGCA.

A further polynucleotide has the sequence of SEQ ID NO. 8 as follows:

5'GAYGGGGAGCAGTGTAACGATGGGCAGAACAAAGATGACCACCATGACGA
CCACCACGATGATCACCATGACGACCATGATGATGATGATGAAACAATGCACT
ATGCCCAGTGTGAAATGGAACCAAACCCTCATATGGCTAGCAGCCTTCACCA
CCATGTCCATGGCAGCATAGAGTTGTCACAGAAGGGTCATGGAGCTGTTTAT
CTAGAACTTCATCTTGTCGGATTCAACACAAGTGAAGACCATGACGACCACCA
TCATGGACTTCATCTGCACATGCTTGGTGACATGTCAGCAGGTTGTGATTCTA
TTGGCGAACTGTACAATGCTCACCCAGAAAAACATGCTGACCCTGGTGACCT
CGGTGACCTGGTTGACGATGATAGGGGCGTGGTTAATGAAGTTCATCATTATG
CTTGGTTGGACATTGATGGTACAGCACCAAACACCGAAGCTCTCATTGGACA
CTCAATGACTATTTTACAAGGGAGTCACACCGATGCTGATACCCCAGCCAGTA
GAATCGCCTGTTGTGTTATTGGTCATGGAAAAGCTCGCCCAGAAACAGCAGC
TGCTCTACATCACGAGCTAGAGGAAGATAAAACTGAGCATTATGCCCATTGTG
ACGTAAGATCTAATACACACCAACCAAAGGCTCTTCATCATCATGTCCACGGA
ACCATCGATTTCAAACAAGTTGGTTATGGTGACCTTGAAGTGTCCTACCATTTA
GAGGGATTTAATGTAAGTGATGACCACAAAGATCATCTCCATGACGTACAGAT
CTACGCCAACGGTGACCTGACCAGTGGATGTGATAACCTCGGTGCTAAATAT
GATCCTCATGAAGATTACCACAGTGAGTTGGGTGATCTAGGAGATATTCACGA
TGATGACCATGGCGTTGTCAATGAAAGCCACAGATATTCCTGGATCAATATCT
TCGGTGATGACAGTGTCCTGGGACGTTCTATTGCCATTCACCAAAGAGACCAT
CTTCATAAAAGTGCCAAAATTGCCTGTTGTGTCATAGGACGTGGACAGAGCCA
TCCAGAAATTGTTCACAGAGCTAAATGTGTTGTCAGACCTAATACAGAATCTAC
TGGTTTACATCACCATGTCTCTGGTTCTATAACATTCGAACAGACCCCTGGAG
GATCAACACATATGACGGCTGATCTCAAAGGATTTAACGTTAGTGAGGACTTG
TCACATCATCGTCATGGTGTGCAGCTCCATGAATGGGGAGATATGTCCCATG
GCTGTCACTCCTTAGGCAGAATGTACCATGGTCATGATGATGCTCATGACCCC
AAAAGACCTGGTGACCTTGGTGATGTTATAGATGATTCCCATGGCATCGTTCA
TTCAACTAGAACCTTTGATCATCTTAATGTTGAAGATCTTAACGCACGTTCCCT
TGTGATTATGCAGGGCGGACATGAGGTCGAGAGTGAGAGGGTTGCTTGCTGT
GTTATAGGACGGGCA**TGA**ATAACCTCACTAGAGTGACTTTGTCTAACATGACA

ATTAACAATTGTATAACTTCGCTAAAA**AATAAAA**CAATGACACAATGNAAAAAA
AAAAAAAAAAAAAAAAAAAAAAAAAAAA3'

with TGA being the opal stop codon and AATAAA the polyadenylation signal.

Variants or homologues of the above polynucleotide sequences also form part of the present invention. Polynucleotide sequences may be aligned, and percentage of identical nucleotides in a specified region may be determined against another sequence, using computer algorithms that are publicly available. Two exemplary algorithms for aligning and identifying the similarity of polynucleotide sequences are the BLASTN and FASTA algorithms. The BLASTN software is available on the NCBI anonymous FTP server (ftp://ncbi.nlm.nih.gov) under /blast/executables/. The BLASTN algorithm version 2.0.4 [Feb-24-1998], set to the default parameters described in the documentation and distributed with the algorithm, is preferred for use in the determination of variants according to the present invention. The use of the BLAST family of algorithms, including BLASTN, is described at NCBI's website at URL http://www.ncbi.nlm.nih.gov/BLAST/newblast.html and in the publication of Altschul, Stephen F, *et al* (1997). "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", *Nucleic Acids Res.* 25:3389-3402. The computer algorithm FASTA is available on the Internet at the ftp site ftp://ftp.virginia.edu.pub/fasta/. Version 2.0u4, February 1996, set to the default parameters described in the documentation and distributed with the algorithm, is preferred for use in the determination of variants according to the present invention. The use of the FASTA algorithm is described in the W R Pearson and D.J. Lipman, "Improved Tools for Biological Sequence Analysis," *Proc. Natl. Acad. Sci. USA 85*:2444-2448 (1988) and W.R. Pearson, "Rapid and Sensitive Sequence Comparison with FASTP and FASTA," *Methods in Enzymology 183*:63-98 (1990).

All sequences identified as above qualify as "variants" as that term is used herein.

Variant polynucleotide sequences will generally hybridize to the recited polynucleotide sequence under stringent conditions. As used herein, "stringent conditions" refers to prewashing in a solution of 6X SSC, 0.2% SDS; hybridizing at 65°C, 6X SSC, 0.2% SDS overnight; followed by two washes of 30 minutes each in 1X SSC, 0.1% SDS at 65°C and two washes of 30 minutes each in 0.2X SSC, 0.1%

SDS at 65°C. Such hybridizable sequences include those which code for the equivalent protein from sources (such as shellfish) other than *P. canaliculus*.

While the above synthetic or recombinant approaches can be taken to produce the protein of the invention, it is however practicable (and indeed presently preferred) to obtain the protein by isolation from *P. canaliculus*. This reflects the applicants' finding that the protein is the dominant protein of the haemolymph of *P. canaliculus* and also that the protein is self-aggregating. It can therefore be isolated in commercially significant quantities direct from the mussel itself. For example, approximately 2 mg of the protein can be obtained per ml of haemolymph.

Once obtained, the protein is readily purified if desired. This will generally involve centrifugation in which the self-aggregating nature of the protein is important. Other approaches to purification (eg. chromatography) can however also be followed.

Furthermore, if viewed as desirable, additional purification steps can be employed using approaches which are standard in this art. These approaches are fully able to deliver a highly pure preparation of the protein.

Once obtained, the protein and/or its active fragments can be formulated into a composition. The composition can be, for example, a therapeutic composition for application as a pharmaceutical, or can be a health or dietary supplement. Again, standard approaches can be taken in formulating such compositions.

Still further, the composition can be a food in which the protein and/or its active fragments are included. This can occur by adding the protein to a pre-prepared foodstuff, or incorporating the protein into a step of the manufacturing process for the food.

The invention will now be described more fully in the following experimental section which is provided for illustrative purposes only.

EXPERIMENTAL

Section 1

A. Materials and Methods

A.1 **Shellfish:** *Perna canaliculus* (the New Zealand green-lipped mussel; the Greenshell™ mussel) were obtained at retail supermarket outlets or from mussel farmers directly; other shellfish species were obtained from retail outlets except for the blue mussel *Mytilis edulis* which was supplied by Sanford's Fisheries (Havelock, New Zealand).

A.2 **Extracts:** Mussel extracts were prepared by homogenising whole, shucked mussels (up to 120 mm length) in a commercial food processor with the addition of 0.02 M sodium phosphate buffer, pH 7.2. Dichloromethane (1/2 volume) was mixed with the aqueous extract, centrifuged at low speed (6000 rpm, GSA rotor, Sorvall RC-5B centrifuge at 4 °C). Polyethylene glycol (PEG) (MW 6000) was added to the aqueous phase to a final concentration of 10% (w/v) and NaCl to 0.5 M and stirred at 4-6 °C overnight. Following low speed centrifugation the PEG-precipitate was resuspended in approximately 1/10 volume of sodium phosphate buffer. After another cycle of low-speed centrifugation the supernatant was centrifuged at high speed (50,000 rpm in a Beckman 60Ti rotor at 4 °C for 60-80 minutes). The resultant pellet was resuspended in a small volume of phosphate buffer and clarified by low speed centrifugation.

A.3 **Polyacrylamide gel electrophoresis:** 12% polyacrylamide gels (8 x10 cm; 1 mm thick) were cast using a prepared stock solution according to the manufacturer's instructions (40% acrylamide/bis solution 37.5:1, Bio-Rad, USA); commercially available 12% gels (Bio-Rad, USA) were also used. Samples (10 µl) were applied to lanes and the gels run at 160 V using a standard Tris/Glycine/SDS buffer (Bio-Rad, catalogue 161-0732) until the bromphenol blue marker reached the bottom of the gel. Gels were stained with BM Fast Stain Coomassie® (Boehringer Mannheim, Germany) and destained as per the manufacturer's instructions.

A.4 **Glycosylation test:** Samples were treated with N-glycosidase F (PNGase F from *Flavobacterium meningosepticum*; Boehringer Mannheim Biochemica, Germany) according to the manufacturer's directions. Treated and untreated samples were run in a standard 12% polyacrylamide gel.

A.5 **Isopycnic gradients:** CsCl (Boehringer Mannhein, Germany) solutions were prepared in 0.1 M sodium phosphate buffer, pH 7.2 and filtered through a 0.22 μm membrane (Acrodisc, Gelman Sciences, USA) to clarify. Two step gradients (1.25 g/cc top layer containing the sample and 1.45 g/cc bottom layer) were prepared as described by Scotti (1985) and centrifuged for approximately 17 hours at 20 °C in a Beckman 70Ti rotor at 30,000 rpm. The resultant gradient was fractionated by inserting a 100 μl glass capillary tube into the gradient and slowly pumping out the contents. UV absorbance was monitored by passing through a Uvicord spectrophotometer (LKB Produkter, Sweden). Fractions were collected and the refractive indices measured using an Abbé refractometer (Bellingham and Stanley, UK) and the density estimated using regression equations according to the method of Scotti (1985).

A.6 **Porous glass chromatography:** Controlled pore glass (CPG 240-80, Sigma Chemical Co., USA) was treated according to the suppliers directions. A 1 cm x 100 cm column (Bio-Rad, USA) was prepared. Samples (1-2 ml) were loaded onto the column and eluted with 0.1 M sodium phosphate buffer, pH 7.2, through a Uvicord spectrophotometer, fractions being collected at regular intervals.

A.7 **Estimation of protein concentration:** Concentrations were estimated using a bovine serum albumin standard (Blot Qualified BSA, Promega, USA) by UV absorption according to the method of Layne (1957) using the equation: mg/ml protein = $1.55*A_{280} - 0.76*A_{260}$. Alternatively, concentration was estimated by the Bradford reaction using reagent supplied by Bio-Rad (USA) at a wavelength of 620 nm..

A.8 High performance liquid chromatography: Reversed-phase HPLC was performed on an HP 1050 Ti-series HPLC (Hewlett Packard, USA) fitted with an analytical 300 Å Vydac C-18 column, 25 cm x 4.6 mm i.d.. The 10 µl sample in water was eluted with a 0-100% acetonitrile in water (v/v) gradient over 60 min and the absorption at 218 and 280 nm was recorded.

B. Results

A light-scattering band was seen after centrifugation of extracts of whole Greenshell™ mussels in CsCl gradients **(Figures 1a and 1b)**. The density of this band was estimated at 1.368 g/cc. A minor band was sometimes observed at approximately 1.390 g/cc. If rebanded in CsCl the 1.390 band yielded two bands - one at 1.390 g/cc and a second at 1.368 g/cc. SDS-PAGE analysis of fractions of either density gave similar polypeptide profiles with a single major band. The molecular weight of the protein by PAGE was estimated as 75,000 (75 kDa) **(Figure 1c)**. Several minor bands of higher molecular weight and an additional minor band of 45 kDa were also seen. The main band (called pernin) at 75 kDa was always at great excess compared to the minor bands. When material from the light-scattering material from CsCl gradients were examined by electron microscopy, particles resembling those of "empty" small RNA viruses were seen **(Figure 2)**. However a UV wavelength scan (data not shown) indicated that little, if any, nucleic acid was present and that the particles were mainly composed of protein. HPLC showed the CsCl band to be composed almost solely of a single species of protein **(Figure 3)**. Since HPLC indicated a high degree of purity, the higher molecular weight polypeptides are presumed to be multimers of pernin. It is likely that the minor, lower molecular weight band is degraded pernin.

Chromatography, on a CPG 240-80 column, of semi-purified extracts, or of material banded in CsCl, showed that the majority of pernin was eluted in the exclusion volume using low molarity phosphate or Tris buffer as the eluent. In contrast, a protein of similar size, bovine serum albumin (68 kDa), was included in the column matrix. It appears, therefore, that pernin does aggregate into large, particle-like structures under certain conditions as suspected from the particles seen in **Figure 2**. HPLC confirmed that pernin from *P. canaliculus* obtained by CPG chromatography was highly purified. Aggregating protein species were also detected in extracts of

other shellfish: the blue mussel *Mytilis edulis*, the oyster *Crassostrea gigas*, and New Zealand pipis *Paphies australis* but not in scallops *Pecten novaezealandiae*. These polypeptides were lower in molecular weight than pernin **(Figure 4a)**. The pernin from *P. canaliculus* is N-glycosylated as shown by a reduction in molecular weight when treated with endoglycosidase-F before PAGE **(Figure 4b)**.

The yield of pernin from whole mussel extractions averaged about 200 μg/mussel. Improved yields of pernin were obtained by extracting haemolymph directly from live *P. canaliculus*. A small notch was made in the shell using a triangular file and a 30 gauge needle inserted into the posterior adductor muscle. From 1 to 5 ml of haemolymph can be withdrawn easily. The haemolymph was spun at low speed (≈1000 *g*) to remove haemocytes and the resulting supernatant processed by ultracentrifugation, for example at 250,000 *g* for 40 minutes, followed by either CPG chromatography eluting with 0.1 M sodium phosphate buffer, pH 7.2, or isopycnic banding in CsCl in phosphate buffer. The pernin obtained in this way appeared no different than that purified from whole mussels and had the advantage of a 30-fold average increase in yield from each mussel. Haemolymph contained around 2 mg/ml (average ≈5-6 mg/mussel) of pernin which is by far the most predominant polypeptide species (**Figure 5a**). The time to purify pernin was reduced from about 5 days to 1 day.

Microsequencing of the N-terminal region and internal fragments generated by chemical and enzymatic cleavage from purified pernin was performed and generated the following sequences of cleavage fragments:

(a) DGEQCNDGQN

(b) QGGHEVESERVACCVIGRA

(c) GQSHPEIVH

(d) YHGHDDA

(e) VVNEVHH.

These sequences code for amino acids as follows:

CODE:	
A	alanine
C	cystine
D	aspartic acid
E	glutamic acid
F	phenylalanine
G	glycine
H	histidine
I	isoleucine
K	lysine
L	leucine
M	methionine
N	asparagine
P	proline
Q	glutamine
R	arginine
S	serine
T	threonine
V	valine
W	tryptophan
Y	tyrosine

The sequence data was then compared with amino acid sequences in searchable computer data bases. Some sequences were found to be of particular interest:

a) a 10 amino acid residue sequence from the N-terminus of pernin (sequence (a) above) showed only homology with an 8 base anti-thrombin protein sequence from terrestrial leeches (data from US Patent 5,455,181 Oct 3, 1995: sequence 10).

Perna canaliculus pernin	**2** GEQCNDGQ **9**
matching amino acids	***G+ CNDGQ***
leech anti-thrombin	**5** GQSCNDGQ **12**

identities: 6/8 (75%) positives: 7/8 (87%);
"+" indicates an equivalent amino acid;
the bolded numerals indicate amino acid position

b) An internal cleavage product (sequence (b) above) was shown to be have homology to the Cu-Zn class of proteins known as "SODs" (superoxide dismutases).

Each of fragments (a) to (e) are part of the larger pernin amino acid sequence:

1	**DGEQCNDGQN**	KDDHHDDHHD	DHHDDHDDDD	ETMHYAQCEM	EPNPHMASSL
5	HHHVHGSIEL	SQKGHGAVYL	ELHLVGFNTS	EDHDDHHHGL	HLHMLGDMSA
0	GCDSIGELYN	AHPEKHADPG	DLGDLVDDDR	G**VVNEVHH**YA	WLDIDGTAPN
5	TEALIGHSMT	ILQGSHTDAD	TPASRIACCV	IGHGKARPET	AAALHHELEE
20	DKTEHYAHCD	VRSNTHQPKA	LHHHVHGTID	FKQVGYGDLE	VSYHLEGFNV
25	SDDHKDHLHD	VQIYANGDLT	SGCDNLGAKY	DPHEDYHSEL	GDLGDIHDDD
30	HGVVNESHRY	SWINIFGDDS	VLGRSIAIHQ	RDHLHKSAKI	ACCVIGR**GQS**
35	**HPEIVH**RAKC	VVRPNTESTG	LHHHVSGSIT	FEQTPGGSTH	MTADLKGFNV
40	SEDLSHHRHG	VQLHEWGDMS	HGCHSLGRMY	**HGHDDA**HDPK	RPGDLGDVID
45	DSHGIVHSTR	TFDHLNVEDL	NARSLVIMQG	**GHEVESERVA**	**CCVIGRA**

(Bold characters indicate directly sequenced fragments (a) to (e)).

Section 2

Anti-thrombin Activity

The possibility that pernin could function as an anti-thrombin agent was examined in a kinetic assay for thrombin inhibition.

Thrombin inhibition assay

Kinetic assays were done using an Accucolor™ Antithrombin III kit (catalogue no. CRS105, Sigma Diagnostics, USA) with the reagents prepared according to the supplier's directions. Standard plasma was supplied by Instrumentation Laboratories (Italy) and used at the recommended dilution of 1/41. Samples of purified mussel protein in water were diluted 9/10 by adding 10X Sigma sample buffer. Heparin was purchased from Instrumentation Laboratories. Thrombin activity was estimated colorimetrically at 405 nm using a chromogenic substrate (H-D-HHT-L-Ala-L-Arg-pNa.2AcOH, catalogue no. A 8058, Sigma, USA) and a Multiskan Biochromatic plate reader (Labsystems, Finland)

This verified that pernin had inhibitory activity. When a purified preparation of pernin was centrifuged through a 30,000 MW exclusion filter **(Figure 5a)**, all the anti-thrombin activity was in the retentate and no detectable activity was present in

the filtrate **(Figure 5b).** The standard serum was diluted 1/41 as recommended for this assay system; the pernin concentration was not determined directly but was in the 1 mg/ml range. From this kinetic data pernin inhibition was estimated to be about 50% of the level of human plasma (approximately 1 mg/ml pernin diluted 9/10 compared with the 1/41 plasma dilution in the standard ATIII assay system). Heparin, a co-factor required for ATIII inhibition of thrombin, was not required for inhibitory action by pernin.

Metal Binding Activity

Hi Trap® Chelating affinity columns (Amersham Pharmacia Biotech, 1ml size) were prepared according to the manufacturer's instructions. The columns were then charged with either 0.1M cupric chloride or zinc chloride before equilibrating in a buffer (0.050M sodium phosphate and 0.5M sodium chloride containing 0.5mM imidazole, pH 7.0). Protein samples purified using CsCl centrifugation were suspended in this buffer and applied to the column using a chromatographic system (Econo System, Bio-Rad Laboratories, USA). Following washing of the column for 5 mins with buffer during which no protein appeared in the eluate, a linear gradient over 20 min at 1 ml/min was used to develop the column using buffer with the imidazole concentration at 100mM from 0-100%. The protein eluted into the gradient being retained longer on the copper chelation column than the zinc. The absorption of the eluate was monitored at 254nM.

Divalent metal ion content of the CsCl purified protein was determined by dissolving the protein in water at 10 mg/ml and analysing metal content by both atomic absorption and plasma emission spectrometry by comparison with a water blank. There was no significant divalent cation content in the protein purified by this method. However, purification by other methods not employing chaotropic agents like CsCl, the high content of histidine coupled with acidic amino acid residues and the likely origin of this protein from a SOD precursor, points to pernin having endogenous metal ions as part of its native structure.

Section 3

Gene Sequencing Method

A suite of non-specific primers called pUZ5 was synthesised by Gibco-BRL for the initial sequencing based on the N-terminal sequence of pernin. The general formula was:

GAY GGN GAR CAR TGY AAY GAY GGN CAR AA

Where Y represents a pyrimidine base, R represents a purine base and N represents any one of the four nucleotide bases. Sequencing was done, initially using pUZ5 and an oligo-dT based "bottom stand" primer from PCR amplified cDNA. Sequencing was done by dye-termination cycle sequencing using "BigDye" prism technology (Applied Biosystems Incorporated, USA) according to their instructions. Products were resolved on an ABI 377 automated sequencer. Following the initial sequencing of approximately 500 base pairs pernin-specific primers were constructed and used to complete the sequencing of the pernin gene.

This provided the following:

GAYGGGGAGCAGTGTAACGATGGGCAGAACAAAGATGACCACCATGACGACCACCACGATGATCA
CCATGACGACCATGATGATGATGATGAAACAATGCACTATGCCCAGTGTGAAATGGAACCAAACC
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TATGCAGGGCGGACATGAGGTCGAGAGTGAGAGGGTTGCTTGCTGTGTTATAGGACGGGCA**TGA**A
TAACCTCACTAGAGTGACTTTGTCTAACATGACAATTAACAATTGTATAACTTCGCTAAAA**AATA**
AAACAATGACACAATGNAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA.

Discussion

The present invention is a novel protein obtainable from *Perna canaliculus*, the New Zealand green-lipped (Greenshell™) mussel. The protein appears to be able to self-aggregate in structures resembling small virus like particles (VLPs) approximately 25 nm in diameter but lacking any nucleic acid. The protein was found in extracts of whole mussels and appears to be the predominant protein in haemolymph. The molecular weight of the protein was estimated to be 75 kDa by PAGE and inferred to be 55 kDa from its polynucleotide encoding sequence but, because of its ability to aggregate, the protein can be sedimented by ultracentrifugation in a short time (e.g. 40 minutes at 250,000 *g*) whereas the monomeric protein would not. Each ml of haemolymph yields, on the average, about 2 mg of pernin. Haemolymph is easily obtained by withdrawing fluid from the posterior adductor muscle of the shellfish which can yield up to 5 ml without obvious harm; it is not necessary to kill the mussel. The haemolymph obtained not only contains high levels of pernin but is quite free of contaminating materials, particularly compared with whole mussel extracts, so purification of pernin is simple. For highly pure preparations of pernin, ultracentrifugation is followed by isopycnic banding in a suitable density gradient medium such as CsCl.

The sequence of the N-terminus of pernin suggested that the protein might have anti-thrombin activity. This was demonstrated in kinetic assays on purified pernin. Since thrombin is a serine protease, pernin also acts as a serine protease inhibitor.

Comparison of the sequences obtained from several cleavage fragments against amino acid sequences in a computer database suggest that in addition to the anti-thrombin activity of pernin, the protein also possesses other activities. One of these is the ability to bind divalent cations such as Zn^{2+} and Cu^{2+}.

INDUSTRIAL APPLICATION

The preferred protein of the invention, pernin, has a number of utilities.

Because of its anti-thrombin activity pernin is potentially useful as an anti-coagulant agent. Thrombin normally acts as a protease which converts fibrinogen in the blood to fibrin. Blood coagulation is counteracted by inhibitors, normally anti-thrombin III (ATIII); pernin has also been shown to inhibit thrombin activity in an ATIII assay system. In contrast to ATIII, whose action is accelerated by the presence of heparin (a sulphated mucopolysaccharide) pernin does not require heparin as a co-factor.

The pernin protein from *P. canaliculus* thus has value as a pharmaceutical. Since it is active as an anticoagulant in its native state it may also be useful as a natural therapeutic agent or health supplement. It is readily obtained as a natural product in high concentrations from mussel haemolymph. To obtain a highly pure preparation it is necessary only to remove haemocytes by centrifugation (or any other suitable method) followed by either ultracentrifugation (since pernin forms aggregates which readily sediment) and resuspension, isopycnic banding in a suitable medium such as CsCl, exclusion filtration through a suitable membrane which retains pernin, or chromatography through a medium such as controlled pore glass of suitable porosity. The result is a highly pure preparation of pernin.

The mussel *P. canaliculus* produces large amounts of the protein naturally, with little cost or effort involved in production, processing or purification.

A further utility of the protein arises from the fact that pernin can be stripped of divalent cations (for example by CsCl isopycnic banding, or pH variation). This allows for the addition of divalent cations of choice (such as Mg^{++}, Cd^{++}, Zn^{++} or Ca^{++}) to the metal stripped pernin. Such a protein, with a modified and pre-selected divalent cation loading, has application in the food and nutraceutical industries.

The ability to bind divalent metal cations also gives rise to applications of the protein in bioremediation and/or cation recovery processes. The divalent cations

can be present as contaminants or pollutants in, for example, a solution, and the solution passed by a substrate to which the protein is bound so that the cations are extracted.

Yet a further utility arises from the fact that the protein is "self-aggregating", and can form into structures resembling empty virus-like particles of approximately 25 nm in diameter. These empty virus-like particles are able to sequester other molecules inside them, with the consequent ability to function as delivery vehicles for those other molecules. Examples of molecules able to be delivered in this manner include pharmaceutically active compounds.

Those persons skilled in the art will understand that the above description is provided by way of illustration only and that the invention is limited only by the appended claims.

REFERENCES

Layne, E. (1957). Spectrophotometric and turbidometric methods for measuring proteins, *Methods in Enzymology* **III**, 447.

Scotti, P.D. (1985). The estimation of virus density in isopycnic cesium chloride gradients. *Journal of Virological Methods* **12**, 149.

CLAIMS:

1. An isolated protein which has a molecular weight of about 55 kDa and an amino acid sequence which includes one or more of the following:

(a) SEQ ID NO. 1

(b) SEQ ID NO. 2

(c) SEQ ID NO. 3

(d) SEQ ID NO. 4

(e) SEQ ID NO. 5

or an active fragment thereof.

2. An isolated protein which comprises the amino acid sequence of SEQ ID NO. 7, or an active fragment thereof.

3. An isolated protein which is obtainable from the haemolymph of *Perna canaliculus* which has an apparent molecular weight of 75 kDa determined by PAGE, or an active fragment thereof.

4. A protein or fragment as claimed in any one of claims 1 to 3 which has activity as:

(i) a serine protease inhibitor; or

(ii) a divalent cation binding agent.

5. A protein or fragment as claimed in claim 4 which has activity as a serine protease inhibitor.

6. A protein or fragment as claimed in claim 4 which has activity as a divalent cation binding agent.

7. A protein which is a functionally equivalent variant of a protein or fragment as claimed in 5 or 6.

8. A protein which is obtainable from a shellfish other than *Perna canaliculus* and which is a functionally equivalent homologue of a protein or fragment as claimed in claim 5 or 6.

9. A polynucleotide encoding a protein or fragment as claimed in any one of claims 1 to 8.

10. A polynucleotide as claimed in claim 9 which comprises the nucleotide sequence of SEQ ID NO. 6 or a variant thereof.

11. A polynucleotide which has the nucleotide sequence of SEQ ID NO. 8.

12. A vector which includes a polynucleotide as claimed in any one of claims 9 to 11.

13. A host cell which expresses a polynucleotide as claimed in any one of claims 9 to 11.

14. A composition which comprises a protein or fragment as claimed in any one of claims 1 to 8.

15. A composition as claimed in claim 14 which is a medicament.

16. A composition as claimed in claim 14 which is a food.

17. A composition as claimed in claim 14 which is a dietary supplement.

18. A dietary supplement as claimed in claim 17 in which said protein or fragment is associated with or bound to at least one divalent cation of dietary significance.

19. A dietary supplement as claimed in claim 18 wherein said divalent metal cation is calcium, magnesium or zinc.

20. A composition as claimed in claim 14 which is a bioremediation agent.

21. A process for obtaining a protein as claimed in claim 3 which comprises the step of centrifuging material containing *Perna canaliculus* haemolymph or an extract thereof and recovering the sedimented protein.

22. A process as claimed in claim 21 wherein said centrifuging step is ultracentrifugation.

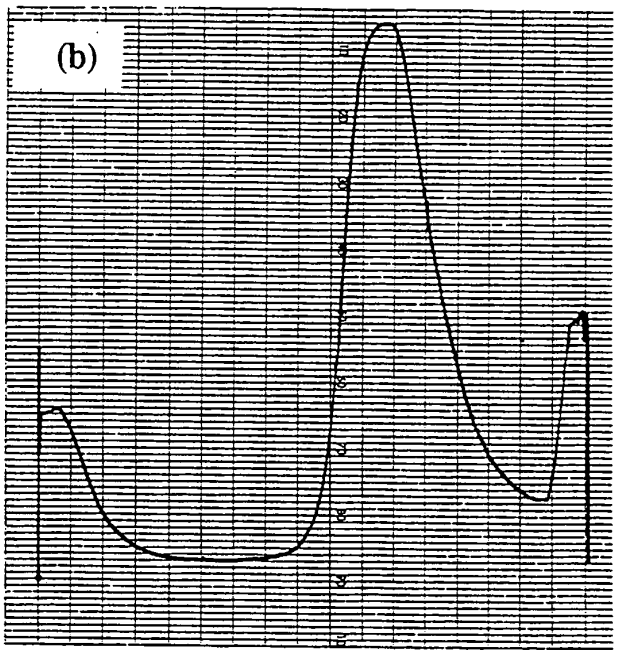
23. A process as claimed in claim 22 wherein said ultracentrifugation is performed for about 40 minutes at about 250,000g.

24. A process as claimed in any one of claims 21 to 23 which includes the preliminary step of extracting haemolymph from *Perna canaliculus*.

09/868760
PCT/NZ99/00227

Figure 1

(a)



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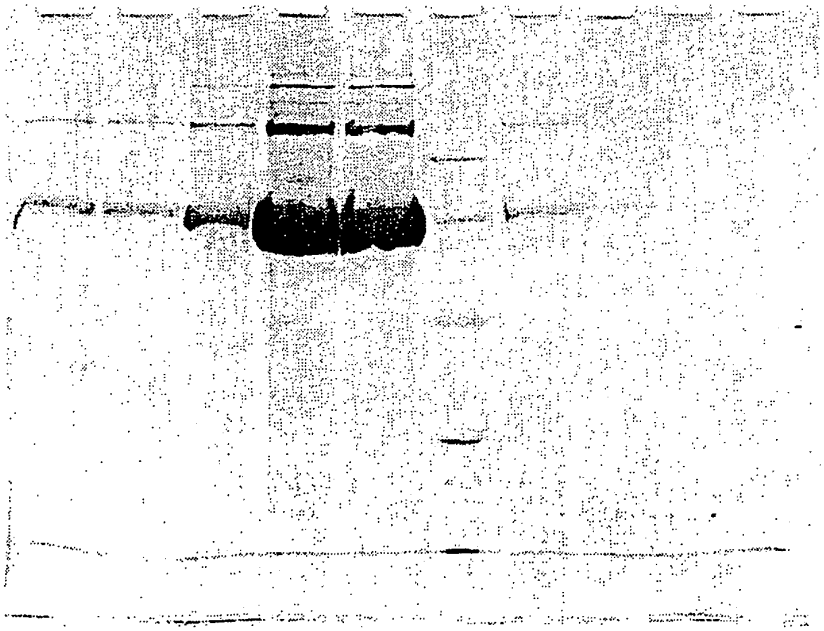

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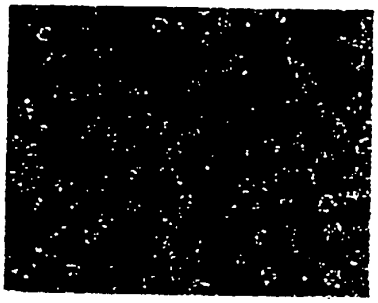

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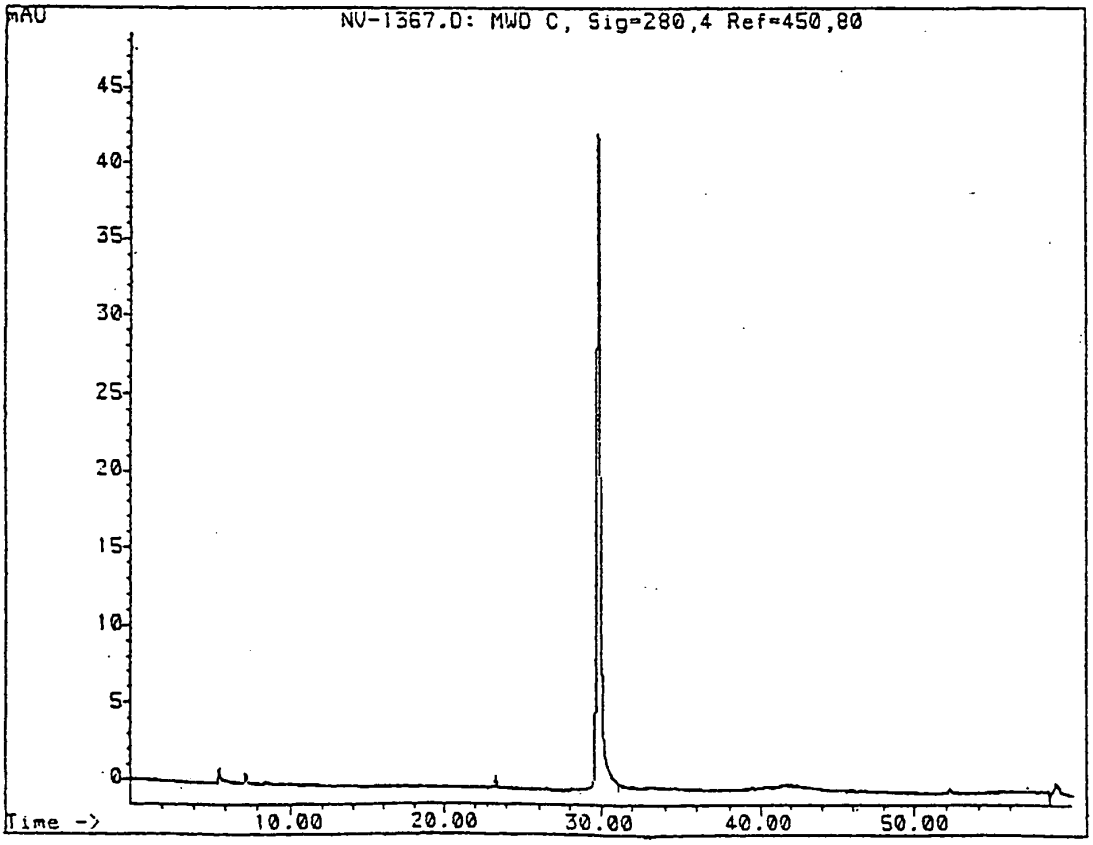

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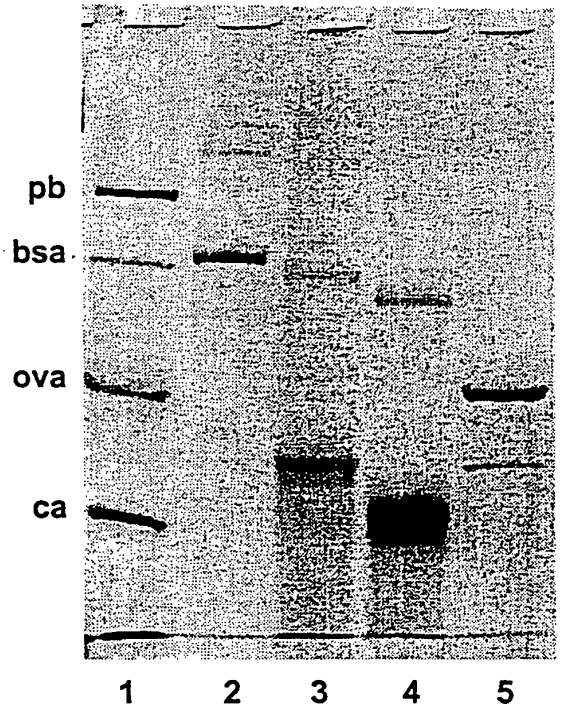

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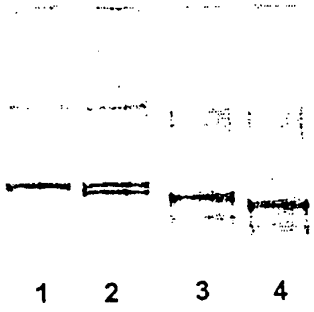
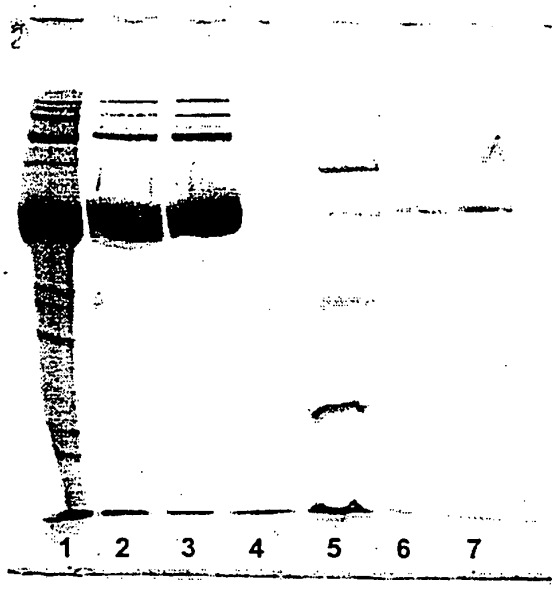


Figure 5

(a)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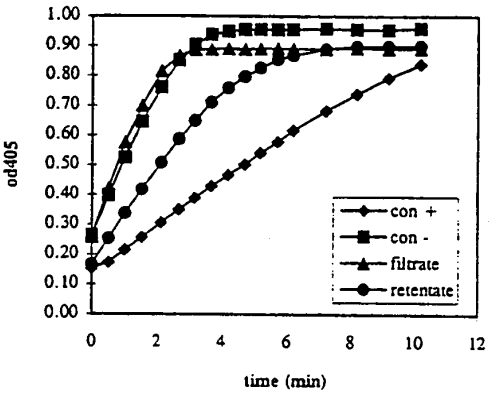
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Asp His His Asp Asp His His Asp Asp His Asp Asp Asp Asp Glu Thr
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His Gly Ala Val Tyr Leu Glu Leu His Leu Val Gly Phe Asn Thr Ser
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gaa gac cat gac gac cac cat cat gga ctt cat ctg cac atg ctt ggt   288
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Cys Asp Asn Leu Gly Ala Lys Tyr Asp Pro His Glu Asp Tyr His Ser
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Glu Leu Gly Asp Leu Gly Asp Ile His Asp Asp Asp His Gly Val Val
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Val Leu Gly Arg Ser Ile Ala Ile His Gln Arg Asp His Leu His Lys
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<222> (1492)..(1494)
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gaaccaaacc ctcatatggc tagcagcctt caccaccatg tccatggcag catagagttg 180

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INTERNATIO EARCH REPORT

International application No.
PCT/NZ99/00227

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: C07K 14/81, C07H 21/04, C12N 15/66, 15/70, 15/74, 15/79, 15/81, 1/21, 1/19, A61K 38/55, 38/57, A61P 7/02, A23J 1/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
STN on-line WPIDS, HCA, BIOSIS, MEDLINE (#thrombin or anti(w)thrombin, serine (w) protease(w)inhibitor) ANGIS(#perna, pernin, mussel, or shellfish or mytilus)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	STN Medline On-line Abstract Accession No. 1999206055, & Barsyte D. et.al., Comparative Biochemistry and Physiology. Part C, Pharmacology, Toxicology and Endocrinology, (1999 Feb) 122 (2) 287-96. See whole abstract, in particular 321-353 and 513-555 nucleotides.	9-11
X	Chemical Abstracts 130:48310, "Sequence and applications in oyster larva settlement of marine Me1A gene", & US 5846531 (Weiner R.) 8 December 1998. See whole abstract.	9-11
X	STN Medline On-line Abstract Accession No.86220028, & Roesijadi G, Enivronemntal Health Persepctives, (1986 Mar) 65 45-8. See whole abstract in particular the 20-25 Kda and 10-12 kDa variants.	8

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 March 2000	27 MAR 2000
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer S.R. IDRUS Telephone No : (02) 6283 2536

INTERNATIONAL SEARCH REPORT

national application No.
PCT/NZ99/00227

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STN Medline On-line Abstract PMID: 2861005, UI: 85229334, & Frazier JM et.al., Comp.Biochem.Physiol.C 1985; 80(2): 257-62	8
X	Chemical Abstracts 94: 62091, "Rapid induction of copper-binding proteins in the gills of metal exposed mussels", & Comp.Biochem.Physiol.C (1980), 67C(2), 215-18. See whole abstract in particular the 12 kDa protein.	8
X	Derwent Abstract Accession No. 87230, Class B04, W0 81/03124 A (PHARMACIA AB) 12 november 1981. See whole abstract in particular "Example".	21-23
X	Derwent Abstract Accession No. 94-163935/20, Class B04, JP 06107682 A (SUNTORY LTD) 19 April 1994. See whole abstract.	21-23
X	Derwent Abstract Accession No. 94-163936/20, Class B04, JP 06107683 A (SUNTORY LTD) 19 April 1994. See whole abstract.	21-23
X	Derwent Abstract Accession No.79466, Class D13(D15), US 4293098 A (SYSTEMS CONSULTANTS) 6 October 1981. See whole abstract, in particular the "Example".	21-23

Supplemental Box
(To be used when the space in any of Boxes I to VIII is not sufficient)

Continuation of Box No: C

The citations included in this Search Report in relation to claim 8 is only a selection found which relate to metal-binding protein from mussells or shell fish other than *Perna canaliculus*.

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
US	5846531	US	5474933				
WO	81/03124	AT	8740	AU	71522/81	DE	3165190
		DK	5771/81	EP	50636	FI	814188
		JP	57500784	SE	8003256	SE	8003253
		US	4550020				

END OF ANNEX

Box III TEXT OF THE ABSTRACT (Continuation of item 5 of the first sheet)

ABSTRACT

The invention provides a protein which exhibits, *inter alia*, anti-thrombin activity and divalent metal cation binding activity. The protein can be readily extracted from the green-lipped mussell, *Perna canaliculus*, and formulated into foodstuffs, nutraceuticals and the like, and has a molecular weight of about 55 kDa and an amino acid sequence which includes one or more of the following:

(a) DGEQCNDGQN (SEQ ID NO.1)

(b) QGGHEVESERVACCVIGRA (SEQ ID NO.2)

(c) GQSHPEIVH (SEQ ID NO.3)

(d) YHGHDDA (SEQ ID NO.4)

(e) VVNEVHH (SEQ ID NO.5)